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Peptide based low molecular weight gelators

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Over the last 12 months a number of papers have been published which shed light on the processes that control the self-assembly of peptides into fibrous hydrogel networks. A number of new properties of dipeptide hydrogels have also been reported. This article highlights recent activity in the area of peptide self-assembly, with a particular focus on tri-peptides, di-peptides and protected amino acids.

Introduction

The last five years have seen a surge in interest in low molecular weight gelators (LMWGs). LMWGs are small molecules that self assemble under the right conditions to form fibrous nanostructures, which can further associate into higher order structures. When self assembled from aqueous solutions the gels typically contain > 99 % water and have been suggested for applications in regenerative tissue engineering, 3-D cell culturing, bio templating, drug delivery, enzyme immobilisation and biosensing¹⁻³.

Of particular interest are LMW peptide hydrogelators which are biomimetic and form translucent hydrogels when added to water⁴. It should be stressed that the peptide does not polymerise in the conventional sense and that the entire material is held together by hydrogen bonding, hydrophobic interactions and π - π stacking. One of the attractions of LMW peptide gelators is that they offer a simple model system for studying the complex problem of peptide self-assembly.⁵ A better understanding of the methods by which peptide chains fold, or misfold, has wide reaching implications for the study of amyloid plaque formation in Alzheimer's patients. In addition a deeper understanding of the self-assembly mechanism is crucial if LMW hydrogels are to be used in real-life applications. In this highlight article, recent investigations are summarised that give a deeper understanding of the self-assembly process. In addition, several recently reported ways of initiating gel formation are discussed.

Hydrophobic interactions and hydrogen bonding in self-assembly.

Despite the range of applications for which LMWGs have been proposed, there is still much to be learned about the specific factors which control gelation and gel strength.

There is currently considerable debate on the relative importance of hydrophobic interactions and π - π stacking interactions to gel formation and gel strength. ⁶⁻⁸ Diphenylalanine (Figure 1, here referred to as H-Phe-Phe-OH to denote the lack of protecting group) was one of the earliest dipeptides studied and has been shown to self-assemble into

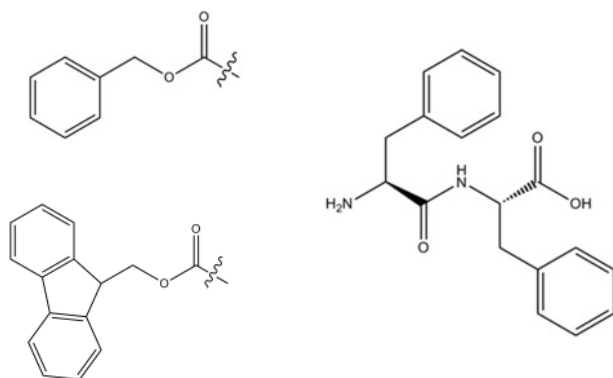


Figure 1. A CBZ protecting group [1]; an Fmoc protecting group [2] and diphenylalanine [3]; diphenylalanine has been denoted H-Phe-Phe-OH to indicate the lack of a protecting group on the N-terminus.

vesicles and peptide nanotubes (although not gels) in solution⁹. In a recent paper, Demirel et al.¹⁰ suggest that H-Phe-Phe-OH assembles mainly due to hydrophobic interactions, evidenced by the fact that no structures form in low dielectric solvents such as THF, toluene and benzene. In contrast, nanotubes or vesicles are formed in high dielectric solvents such as water, methanol, ethanol and acetone. However, Yan et al.¹¹ have shown that H-Phe-Phe-OH forms gels in chloroform or aromatic solvents, such as toluene or xylene, but does not form gels in solvents such as methanol, acetone, cyclohexane, dichloromethane, and N,N-dimethylformamide. This is an interesting discrepancy between observations and may be related to the process by which assembly is carried out.

Efficient hydrogelation appears to require the presence of an aromatic protecting group such as fluorenylmethyloxycarbonyl (Fmoc) or naphthalene (Nap) on the N-terminus of the dipeptide. To give one example, Fmoc-Tyr-OH is a reasonably efficient hydrogelator, whereas CBZ-Tyr-OH and Boc-Tyr-OH (where Boc is a tert-Butyloxycarbonyl protecting group) do not form fibrillar structures in water¹². As mentioned previously H-Phe-Phe-OH will form vesicles or nanotubes in aqueous solution⁹, but Fmoc-Phe-Phe-OH will form extended fibrillar structures that associate to give hydrogels¹³. WAXs and molecular dynamic studies of peptide gels suggest that the Fmoc groups assemble with π - π stacking distances of $< 4 \text{ \AA}$ ¹⁴⁻¹⁵. These studies support the idea that the presence of π - π stacking is important to hydrogel formation. In contrast, a separate study has shown the gelation of Nap-Ala-Val-OH occurs even when a bromo group has been attached to the naphthalene ring in the 6-position¹⁶. It is likely that a large pendant bromine moiety will disrupt π - π stacking between naphthalene rings and yet a gel is still formed. These studies outline that the additional hydrophobicity introduced by the aromatic protecting group may also be important for gel formation, although it should be noted that direct measurements of the distances between the aromatic rings has yet to be carried out for this system.

The role of aromatic side chains on the amino acids has been studied by Yang et al.¹, The authors looked at multi-component supra-molecular hydrogels. They found that gels made from mixtures of Fmoc-Phe-OH and ϵ -Fmoc-Lys-OH were stronger and more elastic than gels composed of Fmoc-Leu-OH and ϵ -Fmoc-Lys-OH. The increase in elasticity was attributed to the increase in π - π stacking introduced by replacing an aliphatic side-chain with an aromatic one. There is, therefore, evidence that the presence of phenyl groups can improve gel strength and elasticity. It is less clear whether π - π stacking between side chains is integral to structure formation. Several studies on the importance

of π - π interactions in protein folding have focused on replacing phenylalanine with cyclohexylalanine (Cha)⁶⁻⁸. This removes aromatic interactions but increases the hydrophobicity of the residue. Bowerman et al.⁸ found that Cha containing peptide sequences were more efficient hydrogelators than equivalent Phe containing peptides, underlining the importance of hydrophobic interactions. Finally Adams et al.¹⁷ have carried out a comprehensive survey of the gelation of a library of Fmoc protected dipeptides. In general, the ability of a dipeptide to form strong gels was found to be correlated to its hydrophobicity. Dipeptides with calculated hydrophobicities below log P 2.8 formed weak and unstable gels, whereas when log P was above 5.5 the dipeptides appeared to be too hydrophobic to form homogeneous gels. At intermediate values of log P (3.4-5.5), all the dipeptides assembled to give gels of similar strengths. The recent studies outlined above suggest that the hydrophobicity of the side chains may play a more important role in the promotion of gelation than has previously been considered.

In addition to hydrophobic interactions, hydrogen bonding is integral to the self assembly process. Several recent publications^{4, 17-18} have confirmed that dipeptides form gels when the carboxylic acid group is protonated (below the pKa of the amino acid). One interesting observation reported in the last 12 months is that the pKa of dipeptides contained within hierarchical structures appears to be considerably higher than the pKa of the free dipeptide in solution.

One theory is that the apparent pKa increase is due to the incorporation of the acid group into a highly hydrophobic environment¹⁴. As mentioned previously, Fmoc-Phe-Phe-OH is an efficient hydrogelator, which will gel water at pH 7. The expected pKa of Fmoc-Phe-Phe-OH in free solution is ~ 3.5 . It has been suggested that after addition of the dipeptide to water an initial assembly process occurs that places the carboxylic groups into a hydrophobic environment, raising the pKa and allowing further hydrogen bonding into fibrils. In a study by Tang et al.¹⁸, two apparent pKa shifts were observed. The first one at pH 10.2-9.5, led to the self-assembly of the dipeptides into a translucent hydrogel composed of paired fibrils. As the pH was further reduced the fibrils were observed to associate to form flat ribbons. A second transition occurred at a pH of 6.2-5.2, below which the ribbons appeared to precipitate from the solution. In a separate study Chen et al.¹⁶ found a higher than expected pKa of 5.9 for Nap-Ala-Val-OH.

An alternative explanation is that the high pKa values arise from stabilisation of the carboxylic acid by a neighbouring molecule. This implies that the high pKa arise from association of molecules at high pH as has been shown for conventional alkyl soaps.¹⁹ This association is thought to arise from the hydrophobicity of the molecules; indeed, a correlation between the logP and pKa has recently been demonstrated for a range of naphthalene-dipeptides.¹⁷

Kinetics of gel formation

Chen et al.¹⁶ have also investigated the link between the kinetics of gel assembly and the final properties of the gel. The pH induced gelation of Nap-Ala-Val-OH was initiated by the slow hydrolysis of glucono- δ -lactone. The kinetics of gel formation were measured by addition of a fluorescent dye, thioflavin-T, the fluorescence of which increases in proportion to the viscosity of the solution. Thioflavin-T is frequently used as an indicator of β -sheet structure formation in polypeptides.

The authors showed that Nap-Ala-Val-OH gelled when the pH dropped below 5.2 and the charge was removed from the C-terminus of the dipeptide. The properties of the resulting gels differed slightly according to the final pH reached. At lower final pHs fewer β -sheet type structures were observed and the fibrils were found to be more rigid. The authors concluded that the speed at which the gel formed is crucial in determining the properties of the material.

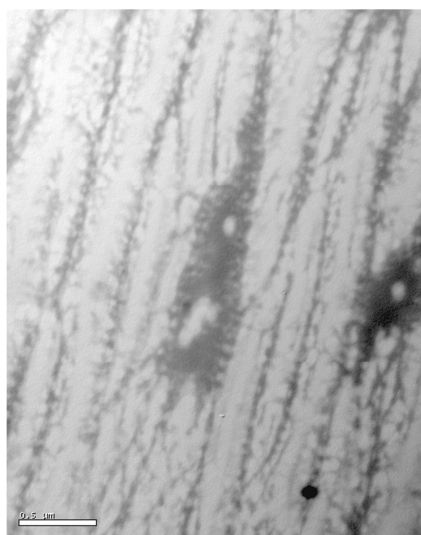


Figure 2. A thin gap spanning LMWG membrane. A surface induced pH drop was used to initiate the gelation of Fmoc-Leu-Gly-OH. For more details see reference [18].

The speed at which the gel formed was also related to gel structure by Johnson et al. in a study of the surface induced assembly of Fmoc-Leu-Gly-OH.²⁰ The kinetics of gel formation were measured directly by Surface Plasmon Resonance Spectroscopy (SPR). It was found that the speed at which the gel formed was directly related to the speed of the pH drop. The final properties of the gel were also dependent on the speed of formation; denser and more heterogeneous gels were formed as the speed of the pH drop increased.

Structure and Sequence

The majority of gel forming dipeptides that have been investigated to date have been shown to self assemble into fibrous networks. Circular dichroism and FTIR show a predominance of β -sheet like structures in the resulting material. Despite the wide variety of dipeptides that form gels, there is still an imperfect understanding of why the presence of some amino-acid residues aids gelation and others cause the dipeptide to crystallise out of solution. Even more interesting is the observation that a simple inversion of the amino-acid at the C- and N- termini can lead to the formation of a completely different material. Cheng et al.¹⁵ investigated the influence of sequence on hydrogel properties. Two protected tri-peptides were compared, Fmoc-Lys-Leu-Val-OH and Fmoc-Val-Leu-Lys-OH. In both cases the lysine residues were protected by Boc groups. The gels were formed in borate buffer and it was discovered that while Fmoc-Val-Leu-Lys-OH assembled into a nematic phase with highly ordered and aligned fibrils, Fmoc-Lys-Leu-Val-OH formed a branched network of fibrils.

The relationship between sequence and structure has been further looked at by Adams et al. in a study of the self-assembled structures formed by Nap-Ala-Gly-OH and Nap-Gly-Ala-OH. A pH drop induced gelation of Nap-Gly-Ala-OH but crystallisation of Nap-Ala-Gly-OH. Computational modelling

based on x-ray diffraction studies suggested that the different structures could be due to differences in conformational and hydrogen bonding preferences between the two molecules²¹. Finally Debnath et al. have investigated the antimicrobial properties of Fmoc protected di-peptides that are functionalised with a pyridinium moiety at the C-terminus. Several of the compounds screened proved to be strongly bacteriocidal, with the most active Fmoc-protected dipeptides containing an L-phenylalanine residue at the N-terminus. Interestingly swapping L-phenylalanine from the N- to the C-terminus of the molecule greatly lowered its anti-microbial properties. It has been suggested that the increased anti-microbial effect could be seen when the phenyl side group is adjacent to the Fmoc group as this conformation leads to 'optimal hydrophobicity'.²²

New methods for initiating the self-assembly of dipeptide hydrogels.

A variety of new gelation methods have been developed and published in the last 12 months. An interesting paper by Xie et al.²³ describes the spontaneous gelation of supersaturated solutions of cyclic dipeptides upon the introduction of sheer forces. The supersaturated solutions were meta-stable and remained liquid for days, but gelled as soon as the container was perturbed. In our own work,²⁰ we have shown that electrochemical methods can be used to grow nanometre thick dipeptide hydrogel films and gap spanning hydrogel membranes. The membranes have the potential to open up the gels to a range of applications where bio molecules immobilized in the membrane are in contact with reservoirs on both sides. Wu et al.²⁴ have used sonication to induce supra-molecular structuring of peptide based gelators in the presence of NaYF₄ nanoparticles. Finally enzyme triggered gelation is an exciting area that continues to be developed²⁵, a recent review of which can be found in reference 22 .

Emerging research into the properties and applications of dipeptide hydrogels.

Some interesting new applications of peptide hydrogels have been investigated in the last year. Jayawarna et al. have reported the fine tuning of gels for use as scaffolds for cell growth. Fmoc-Ser-OH, Fmoc-Lys-OH or Fmoc-Glu-OH were mixed with Fmoc-Phe-Phe-OH and cell culture media to create a range of gels. The presence of a particular Fmoc-amino acid in the gel was found to affect the visco-elastic properties of the gel as well as introducing different chemical functionality. The authors showed that they could tune the gel properties to improve the growth and proliferation of bovine chondrocytes, mouse 3T3 fibroblasts and human dermal fibroblasts³. In a second report by the group mixed gels of Fmoc-Phe-Phe-OH and Fmoc-Arg-Gly-Asp-OH were shown to be promising 3D-cell scaffolds for the growth of encapsulated human dermal fibroblasts²⁶. Both of these reports show the potential of LMWGs as low cost and simple matrices that can be tuned to support the growth of a range of cells.

Fig. 3 (reproduced from reference 27) (a) Structure of naphthalene-diphenylalanine (I) and dansyl derivative (II). (b) I forms a transparent self-supporting hydrogel at a concentration of 2.2 mM and a pH of 4 (left). A transparent self-supporting gel was also formed in the presence of II (0.084 mM). (c) Energy transfer occurs between I and II (white rectangle) hosted within the fibres that form via hydrogen-bonding between dipeptides leading to emission at 485 nm in addition to emission at 355 nm from I alone.

Interesting new properties of peptide based hydrogels have been investigated by a number of groups. Xu et al.¹⁴ have used impedance spectroscopy to study the conductivity of peptide fibrils formed by the enzyme triggered self-assembly of Fmoc-Leu-Leu-Leu-OH. The conductivity of gel films was measured in both air and under vacuum and was found to increase linearly with peptide concentration. The conductivity in air was found to be of the order of 100 k Ω /sq and in wet gels both electron transport and ion diffusion were observed. While the conductivity is still relatively low, this work opens the possibility of using self-assembled peptide nanowires to connect cells or proteins to electric circuits. Chen et al.²⁷ prepared multi-component hydrogels containing donor and acceptor chromophores. The naphthalene protecting group on Nap-Phe-Phe-OH could undergo energy transfer to both dansyl and anthracene acceptors in an organic solvent free hydrogel. This could lead to peptide hydrogels being used as light harvesting materials. Finally Amdursky et al. have studied the optical properties of Fmoc-Phe-Phe-OH and have reported quantum confinement effects. At di-peptide concentrations below which gels are able to form the molecules appeared to organise into structures that contained nano-sized crystalline regions. These nanostructures showed an absorption spectrum very similar to that of quantum dots. At higher dipeptide concentrations, hydrogels were formed that showed quantum-well like confinement, with a sharp photoluminescence peak in the UV28. Both of these reports open the way for the use of LMW gels in solar cells or light emitting devices. The potential to use cheap organic molecules in an aqueous environment for light harvesting or emission is an exciting development that will no doubt be investigated further in the future.

Conclusions

This highlight article has summarised some of the recent literature in the field of low molecular weight peptide hydrogelators, which form extended fibrous structures through a combination of hydrogen bonding and hydrophobic interactions. Over the last 12 months, progress has been made in the understanding of the mechanism and the kinetics of the self-assembly process. In addition a number of interesting new applications for self-assembled peptide hydrogels have been reported.

Notes and references

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